

Detection of autoantibodies against alpha-2-macroglobulin-like 1 in paraneoplastic pemphigus sera utilizing novel green fluorescent protein- based immunoassays

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Highlights

- The majority of PNP patients develop autoantibodies against A2ML1
- The biological function of A2ML1 in the epidermis remains incompletely understood
- Current diagnostic methods in PNP are inadequate to detect anti-A2ML1 antibodies
- Here we developed sensitive and quantitative assays to detect anti-A2ML1 antibodies
- Our approach can be applied for the characterization of antibodies against various antigens

Summary

Background. Paraneoplastic pemphigus (PNP) is a devastating autoimmune multiorgan syndrome associated with autoantibodies against several autoantigens, including the alpha-2-macroglobulin-like-1 (A2ML1). A2ML1 is recognized by up to 70% of PNP sera. The currently recommended techniques for serological diagnosis of PNP are inadequate to detect anti-A2ML1 antibodies.

Objectives. To develop novel assays which allow to easily and reliably detect anti-A2ML1 autoantibodies in PNP sera.

Methods. We produced full-length A2ML1 in fusion with enhanced green fluorescent protein (EGFP-A2ML1) in transfected human embryonic kidney 293T cells. The recombinant protein was used as fluorescent ligand for immunoprecipitation studies. We further developed an enzyme-linked immunosorbent assay (ELISA) by immobilizing EGFP-A2ML1 on 96-well plates.

Results. A2ML1-positive PNP sera were able to immunoprecipitate EGFP-A2ML1. Direct measurement of fluorescence in immunoprecipitates correlates with the relative levels of anti-A2ML1 antibodies in the PNP sera. By the novel ELISA, based on the determined best cut-off value, 61% of the tested 36 PNP sera were A2ML1 positive with a specificity of 88.9% and a sensitivity of 95%. The 20 tested normal sera (NHS) were negative, while 2 (10%) of 20 pemphigus vulgaris and 3 (15%) of 20 bullous pemphigoid sera showed borderline values.

Conclusions. Our novel immunoassays enable rapid stratification of PNP patients. The novel green fluorescent protein-based ELISA utilizing an active eukaryotic A2ML1 is highly sensitive and reliable and, hence, is useful for a better understanding of the immunological background of PNP. This approach may be easily applied for the rapid detection of antibodies to various other antigens.

Keywords: diagnostic immunoassays, autoimmunity, paraneoplastic pemphigus, alpha-2-macroglobulin-like 1

Introduction

Paraneoplastic pemphigus (PNP), also termed paraneoplastic autoimmune multi-organ syndrome (PAMS), is a severe autoimmune blistering disease of the skin and mucosae associated with an underlying neoplasia. PNP patients have circulating autoantibodies binding to components of stratified as well as non-stratified epithelia [1, 2, 3]. Specifically, PNP autoantibodies typically bind to members of the plakin family of proteins, including envoplakin, periplakin, desmoplakin, and, more rarely, the bullous pemphigoid antigen 230 and plectin [1, 4-14]. In addition, PNP sera contain autoantibodies directed against the desmosomal components desmoglein 3 and desmoglein 1 in about 65% and 25% of cases, as well as against desmocollin 2 and desmocollin 3 in 40% and 60% of the PNP sera, respectively [15, 16]. Ample evidence exists that autoantibodies against desmogleins play a central role in disrupting cell adhesion, causing mucocutaneous blistering [7]. While the exact role of antibodies directed against plakins remains unclear, it is likely that CD8⁺ cytotoxic lymphocytes and other mononuclear contribute to tissue damage [2, 3, 17].

We have previously identified that the 170 kDa protein (p170) immunoprecipitated by PNP sera corresponds to the broad range protease inhibitor alpha-2-macroglobulin-like 1 (A2ML1) [18, 19]. A2ML1 expression in the epidermis is restricted to the granular layer and its biological function remains incompletely understood [18, 20].

A number of observations indicate that autoantibodies against A2ML1 may be pathogenic and may contribute to tissue damage. First, in a significant number of reported PNP patients, autoantibodies against A2ML1 are found alone or together with few additional autoantibodies [18, 21]. Second, PNP sera binding to A2ML1 can be detected already at an early

stage of the disease [18, 22, 23]. Third, A2ML1 is not only expressed in skin but also in other organs affected in PNP (Hs.620532, NCBI Unigene, EST profile viewer) [18]. Finally, we have provided evidence that polyclonal anti-A2ML1 antibodies cause decreased cellular adhesion of cultured keratinocytes and an increase of plasmin activity in vitro [19]. In addition to envoplakin, periplakin and desmocollins, A2ML1 is one of the most characteristically recognized autoantigens in PNP [1, 18, 19]. Nevertheless, standard immunoblotting and rat bladder indirect immunofluorescence (IIF) studies, the laboratory techniques routinely recommended for an easy and rapid diagnosis of PNP, are unable to detect anti-A2ML1 antibodies in PNP [21]. Therefore, novel tests to specifically and sensitively detect anti-A2ML1 antibodies are needed. Such tests are of crucial importance to improve the diagnosis of PNP and to better delineate the biological role of A2ML1. In this study, we sought to develop sensitive and quantitative assays to detect anti-A2ML1 autoantibodies in human PNP sera.

Materials and methods

Patient and control sera

Serum samples from 36 PNP patients were analysed in this study. The diagnosis of PNP was made based on consistent clinical, histological and immunopathological findings [1, 2]. Serum samples from 20 bullous pemphigoid (BP) and 20 pemphigus vulgaris (PV) patients as well as 20 normal human serum samples were included as control.

PNP, BP and PV sera were collected from patients managed in Germany and Japan according to the local ethical rules, before the research was started.

Normal human serum samples were acquired from 20 healthy blood donors of the local regional Swiss blood bank.

The ethical committee of the Canton of Bern approved the final study.

Cloning

We previously cloned the extracellular domain of desmocollin 3 (DSC3) in fusion with enhanced green fluorescent protein (DSC3 ECD-EGFP) for other purposes, using plasmid pcDL-SRa196 containing DSC3 [16]. The extracellular domain was amplified using this plasmid as template by PCR using 5'CTCCTCCTCGAGATGGCCGCCGCTGG and 5'GAGGAGGGATCCACTGCCGCCACCACTGCCGCCACCTTTTCCAAGTATTACTCCTGTACTCCTTGAAG primers and cloned into vector v127 encoding for EGFP.

To clone a secreted recombinant form of EGFP (sEGFP), as negative control, the DSC3 extracellular domain was removed by using restriction enzymes, leaving the signal sequence and the propeptide (v143).

Clone OHS5893-202503807 (AccNo BC172370) encoding for A2ML1 full-length cDNA was purchased from Dharmacon (Lafayette, Colorado, USA). A2ML1 was amplified by PCR using 5'CTCCTCAAGCTTGCTAGCGAAGAACTTCCAAACTACCTGGTG and 5'GAGAAGCCAAGGGAAACCTG primers and introduced in v143 in N-terminal fusion with EGFP to generate EGFP-A2ML1.

All generated plasmids were verified by sequencing (Microsynth, Balgach, Switzerland).

Cell culture and transfection

Human embryonic kidney (HEK) cells 293T (American Type Culture Collection, Manassas, Virginia, USA) cells were grown in DMEM supplemented with fetal bovine serum (FBS) 10%, penicillin, and streptomycin (Invitrogen, Carlsbad, California, USA). HEK 293T cells were transfected according to the calcium phosphate method.

Preparation of proteins from cell culture medium

The culture medium of HEK 293T transfected cells was collected, cell debris were removed by centrifugation and proteins were precipitated with the addition of 10% v/v trichloroacetic acid in ice for 30 min. The pellet was collected after centrifugation for 20 min at 20000 RCF, washed with ice-cold acetone and resuspended in Laemmli sample buffer supplemented with 6M urea.

Protein separation and protease activity assay

Protein reducing denaturation was performed in Laemmli buffer, in which samples were not boiled. Proteins were then fractionated on 10% SDS-PAGE gel, which was scanned for fluorescence with Typhoon 9400 imager instrument (GE Healthcare, Chicago, Illinois, USA).

EGFP-A2ML1 inhibitory activity towards chymotrypsin was evaluated using the commercial fluorometric protease activity assay Kit (Abcam, Cambridge, UK), according to the manufacturer's protocol. Casein TAMRA-conjugated (LuBioScience, Zürich, Switzerland) was used as a substrate.

Fluorescence released after the enzymatic reaction was measured at Ex 540 nm/Em 590 nm with Tecan Spark 10M.

EGFP fusion protein concentration was calculated as previously described [24].

Fluorescent immunoprecipitation (IP)

Patient sera and controls (30 µl) were incubated with 50 µl of resuspended protein A-Sepharose resin (GE Healthcare, Chicago, Illinois, USA) at 4°C for 1 hour under agitation. After discarding the unbound fraction, the beads were washed two times with phosphate buffered saline (PBS)

and incubated for 1 hour at 4°C under agitation with the culture medium of 293T transfected cells expressing sEGFP or EGFP-A2ML1. After washing again as described before, the fluorescence associated with the beads was measured at Ex 485 nm/Em 530 nm with Tecan Spark 10M.

Enzyme-linked immunosorbent assay (ELISA)

The crude medium of 293T transiently transfected cells expressing sEGFP or EGFP-A2ML1 was used to coat ELISA plates (Nunc Maxi sorp plates, Thermofisher, Waltham, Massachusetts, USA) by incubation at room temperature (RT) for 2 hours under agitation.

Wells were washed two times with PBS and blocked overnight with PBS+5% skim milk at 4°C. Serum samples from patients and controls, diluted 1:10 in PBS+5% skim milk, were subsequently added to the microplate and incubated for 2 hours at room temperature under agitation.

After washing three times with PBS, a Horseradish peroxidase-labelled goat anti-human IgG (Thermofisher, Waltham, Massachusetts, USA), diluted 1:2000 according to the manufacturers' instructions, was applied for 1h at room temperature and used for detection.

Three washing steps with PBS were followed by the addition of tetramethylbenzidine (TMB) to each well and absorbance was measured with Tecan Spark 10M at 450 nm after stopping the enzymatic reaction with HCl.

Statistics

All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, California, USA). A receiver operating characteristic (ROC) curve was calculated in order to explore the diagnostic performance of the ELISA.

Results

Expression and characterization of EGFP-A2ML1

EGFP is a versatile reporter protein useful for monitoring several aspects of recombinant protein production [24]. Using transiently transfected HEK 293T cells we produced EGFP-A2ML1 and sEGFP as control, which were secreted into the culture medium. The expression was verified by SDS-PAGE in gel fluorescence detection of EGFP-tagged proteins (Fig. 1 a). The use of human cells ensures the proper post-translational glycosylation of the expressed proteins, whereas the presence of EGFP allows to easily detect and measure the concentration of the expressed proteins in any environment [24].

Since A2ML1 is able to inhibit chymotrypsin [24], we investigated whether the produced EGFP-A2ML1 has the expected biological activity. For this purpose, we used a commercial protease activity assay which uses casein, as a generic protease substrate, labelled with a red fluorescent dye. EGFP-A2ML1 was able to inhibit chymotrypsin in a dose dependant manner, an observation implying that the chimeric protein is biologically active and antigenically intact (Fig. 1 b-c).

EGFP-A2ML1 allows an easy and rapid detection of autoantibodies in human PNP sera by immunoprecipitating A2ML1

We next determined whether EGFP-A2ML1 from the culture medium of transfected HEK 293T cells could be detected in immunoprecipitation (IP) by PNP sera. Four well characterized A2ML1-positive PNP sera (PNP 5, 6, 7 and 8) and two NHS from healthy donor (NHS1 and NHS2) were used to immunoprecipitate either sEGFP or EGFP-A2ML1. Direct fluorescence quantification of

EGFP-A2ML1 in the obtained immunoprecipitates identified A2ML1-positive versus A2ML1-negative PNP sera. All four A2ML1-positive PNP sera immunoprecipitated EGFP-A2ML1 whereas the two NHS did not (Fig. 2). Fluorescence intensity correlates with the anti-A2ML1 antibody relative levels present in the samples (Supplementary, Figure 4). Hence, this modified IP approach more easily and more rapidly allows to identify PNP sera reactive with A2ML1 when compared to the standard IP techniques [1, 4, 21].

EGFP-A2ML1 ELISA

To simplify the screening of large numbers of samples, we next established an ELISA to test and quantify the reactivity of human sera against A2ML1, using the EGFP-A2ML1 protein. The sEGFP and EGFP-A2ML1 recombinant forms present in the culture medium of transfected HEK 293T cells were immobilized on ELISA plates. The plates were subsequently incubated with sera from patients with PNP (n=36), PV (n=20), BP (n=20) as well as NHS (n=20). Two different dilutions of sera (1:10 and 1:100) were tested, but the 1:10 dilution produced the best outcome in terms of reproducibility and signal intensity (Supplementary, Figure 5).

To determine the diagnostic performance of this test a receiver operating characteristic (ROC) analysis was performed (Fig. 3 a), using as truly positive/negative some of the previously mentioned PNP sera which were already extensively tested in past publications [18, 21]. The ELISA showed a sensitivity of 95%, a specificity of 88.9% and an area under the curve (AUC) of 0.956, with a 95% confidence interval. Based on these parameters, the cut-off was set at 0.2 absorbance units at 450 nm. With this ELISA method and the presently set cut-off value, 22 of 36 (61%) PNP sera were positive, 2 of 20 (10%) PV patients and 3 of 20 (15%) BP patients were positive with borderline values, whereas none of the tested NHS was positive (Fig. 3 b). These results show a high correlation with results obtained in previously used methods; i.e., IP of either

radioactive or non-radioactive differentiated keratinocytes extracts, as well as immunofluorescence microscopy of transfected cells expressing A2ML1 [17, 21].

Discussion

The diagnosis of PNP may be difficult, since it often presents with polymorphous mucocutaneous features, mimicking PV, BP, erythema multiforme, Stevens-Johnson syndrome and lichen planus [1, 2]. Furthermore, physicians are almost invariably not familiar with this disorder, because less than 500 cases of this condition have been reported so far worldwide [3].

The detection of distinct autoantibodies with appropriate serological tests is therefore of utmost importance to diagnose PNP patients. Proper diagnosis has a critical impact on patient's care and management and has significant prognostic value. Rat bladder IIF and immunoblotting are the most widely used techniques [21]. However, these techniques are unable to detect the most characteristic autoantigen of PNP, A2ML1, the identification of which has represented a challenging task for two decades [17]. Furthermore, there are PNP cases which show immune reactivity with only A2ML1, which may thus be entirely missed by the recommended combination of standard assays [20]. The identification of anti-A2ML1 autoantibodies in PNP sera is presently based on immunoprecipitation of radioactive or non-radioactive differentiated human keratinocyte extracts followed by polyacrylamide gel autoradiography or immunoblotting, respectively [1, 4, 5, 9, 18, 21]. These methods are labour-intensive, costly and prone to false results. Faster, easier and reliable diagnostic tools are thus needed. The use of EGFP-A2ML1 enabled us to develop new methods for serological detection of anti-A2ML1 autoantibodies.

Our results demonstrate that direct fluorescence quantification of EGFP-A2ML1 IPs can be easily used to rapidly identify A2ML1-positive PNP sera, greatly facilitating the assessment of reactivity with A2ML1 of PNP sera in comparison with the presently used IP methods [21].

Furthermore, fluorescence intensity seems to correlate with the anti-A2ML1 antibody relative levels present in the samples, a contention supported by the observed linear correlation between the EGFP-A2ML1-based IP and ELISA findings.

The newly developed ELISA using a soluble green fluorescent protein-tagged A2ML1 allows us to easily identify A2ML1-positive sera and further simplifies the screening of a large number of human sera. This newly developed ELISA has a sensitivity of 95% and a specificity of 88.9% for the detection of circulating anti-A2ML1 antibodies in PNP sera. It should be noted that these results are limited by the fact that we used retrospectively collected well-characterized PNP serum samples, which were almost invariably anti-A2ML1 positive samples, as assessed by a combination of different techniques [18, 19, 21]. Therefore, prospective studies are needed to exactly define the diagnostic power of this novel assay with longitudinal analyses of selected patients to be able to correlate IgG anti-A2ML1 serum concentrations with the disease status. It is worth mentioning that the crude supernatant of transfected 293T cells was used to develop these tests, without carrying out a protein purification procedure. The latter was found to be problematic and inefficient because of the low protein expression. Two of 20 (10%) PV sera and 3 of 20 (15%) BP sera have been found to be A2ML1-positive with borderline values. An explanation for this finding may be the high sensitivity of the test using a biologically active eukaryotic protein. The epitope spreading phenomenon, described in various autoimmune bullous diseases of the skin may be responsible for the development of anti-A2ML1 antibodies at low titers during the disease course. These speculations need to be verified by screening large cohorts of patients and healthy controls. An increasing number of patients suffering from atypical forms of pemphigus have been recently reported: their correct classification and adequate management critically depend on the characterization of their immunological reactivity. Since clinicopathological features in combination with standard immunofluorescence microscopy and/or immunoblotting studies are not always conclusive, our novel diagnostic

assays fill an important practical gap in the serological diagnosis of PNP. These immunoassays seem to rapidly and easily allow to identify A2ML1 reactivity as useful diagnostic biomarker.

Although we have obtained evidence indicating that anti-A2ML1 antibodies cause decreased cellular adhesion of cultured keratinocytes and an increase of plasmin activity in vitro and may be thus pathogenic in vivo, little is known about the exact biological functions of A2ML1, a broad range protease inhibitor predominantly expressed in the granular cell layers of the epidermis. A2ML1 shows homology with alpha-2-macroglobulin suggesting a possible involvement in innate immunity [25]. However, based on the ability of A2ML1 to covalently bind to kallikrein 7 and other serine proteases cascades in vitro, it is likely to play a role in the maintenance of skin homeostasis [20, 26].

We anticipate that the developed novel immunoassays will provide useful tools for the further study of the biological function of A2ML1 in both maintenance of tissue homeostasis and in the pathogenesis of PNP. In fact, we have found that the soluble EGFP-A2ML1 fusion protein is capable of inhibiting the proteolytic activity of chymotrypsin in a dose dependant manner. It also provides a useful tool to further investigate the effect of anti-A2ML1 antibodies in vitro models. EGFP-A2ML1 is biologically active and is immunoprecipitated by PNP sera, indicating that the EGFP tag does not interfere with the proper folding and function of A2ML1.

Finally, this study has important implications beyond the serological characterization of PNP patients. Our approach to establish efficient assays based on green fluorescent protein to quantify the reactivity of human sera against a defined protein autoantigen can easily be translated and applied to a broad spectrum of autoimmune diseases. Once the relevant EGFP-tagged autoantigen is produced, fluorescence immunoprecipitation and ELISA analyses can be performed in well-defined conditions and are quantitative. While fluorescence immunoprecipitation is better suited for the analysis of few sera, the ELISA provides a rapid and useful tool for large-scale screening of sera.

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Figure legends

Figure 1. EGFP-A2ML1 secreted in the culture medium of transfected human embryonic kidney (HEK) 293T cells inhibits chymotrypsin in a dose-dependant manner. (a) Culture medium of transfected HEK 293T cells producing EGFP-A2ML1 or sEGFP was size-fractionated on 10% SDS-PAGE, which was scanned for fluorescence at Ex 485 nm/Em 530. **(b)** A fluorometric protease activity assay kit with casein-TAMRA conjugated as substrate was used to test the inhibitory activity of EGFP-A2ML1 on chymotrypsin. Red fluorescence released by casein cleavage was measured with a fluorometer at Ex 540 nm/Em 590 nm. Data are representative of three independent experiments. Statistics: One-way ANOVA, $p < 0.0001$ **(c)** Serial dilutions of EGFP-A2ML1 were made to obtain an inhibition curve.

EGFP, enhanced green fluorescent protein; A2ML1, alpha-2-macroglobulin-like 1; RFU, relative fluorescence unit; SC, substrate control (protease substrate, casein TAMRA-conjugated); PC, positive control (trypsin and protease substrate); VC, vehicle control (PBS).

Figure 2. Immunoprecipitation (IP) of EGFP-A2ML1 with paraneoplastic pemphigus (PNP) sera.

IPs were performed with the culture medium of transfected HEK 293T cells producing either EGFP-A2ML1 or sEGFP. Fluorescence associated with the protein A-Sepharose beads was measured with a fluorometer at Ex 485 nm/Em 530. The sEGFP background was subtracted from the EGFP-A2ML1 values. NHS, normal serum; RFU, relative fluorescence unit.

Figure 3. EGFP-A2ML1 enzyme-linked immunosorbent assay. (a) Receiver operating characteristic (ROC) analysis of EGFP-A2ML1 results. AUC, area under the ROC curve. **(b)** Sera from pemphigus vulgaris (PV), bullous pemphigoid (BP) and paraneoplastic pemphigus (PNP) patients, as well as normal human sera from healthy donors (NHS) were analyzed by the ELISA based on EGFP-A2ML1. Absorbance values measured at 450 nm are presented by the plotted data points. The cut-off value is indicated by the dashed line and set at absorbance 0.2. The sEGFP background was subtracted from the EGFP-A2ML1 values.

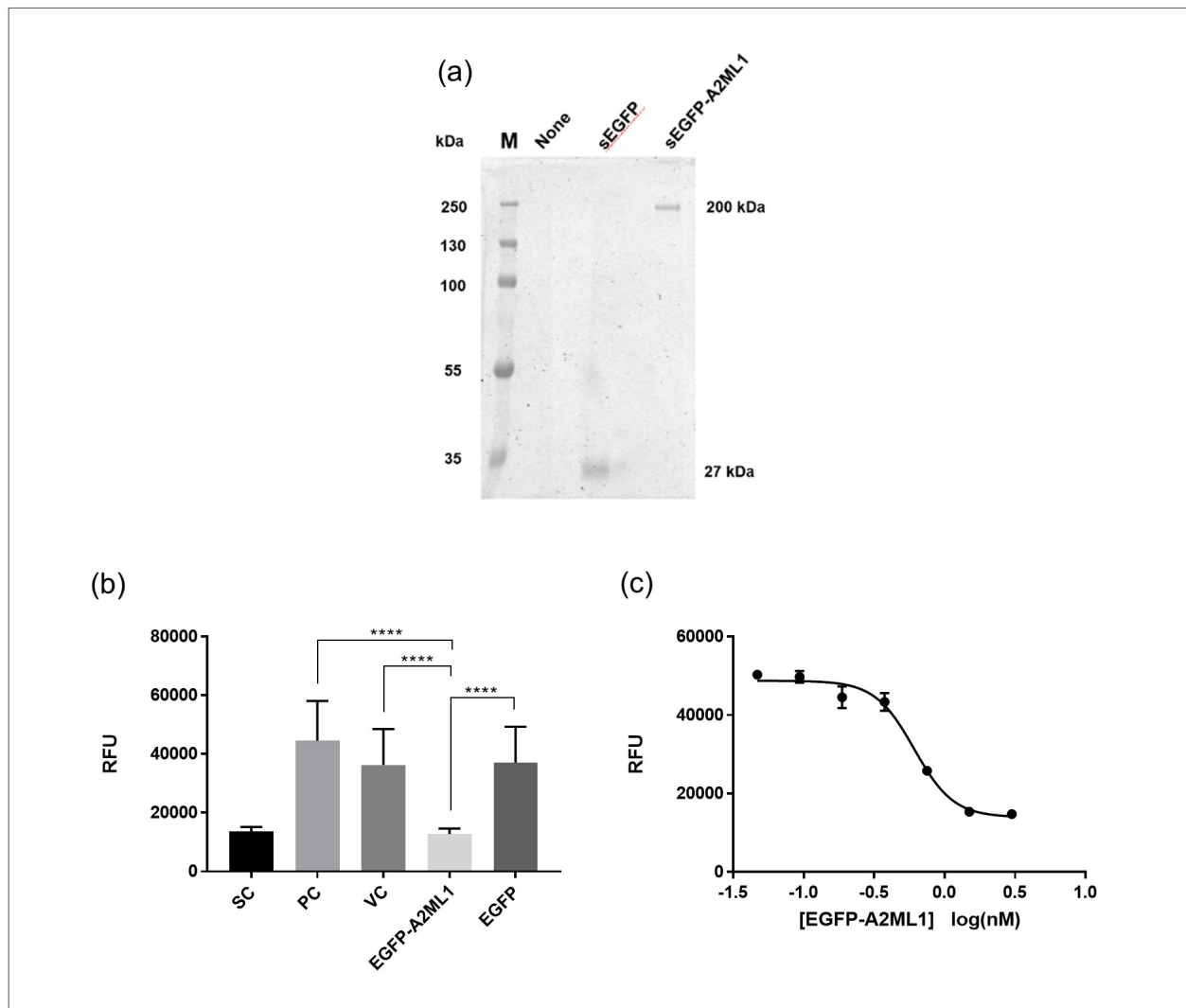


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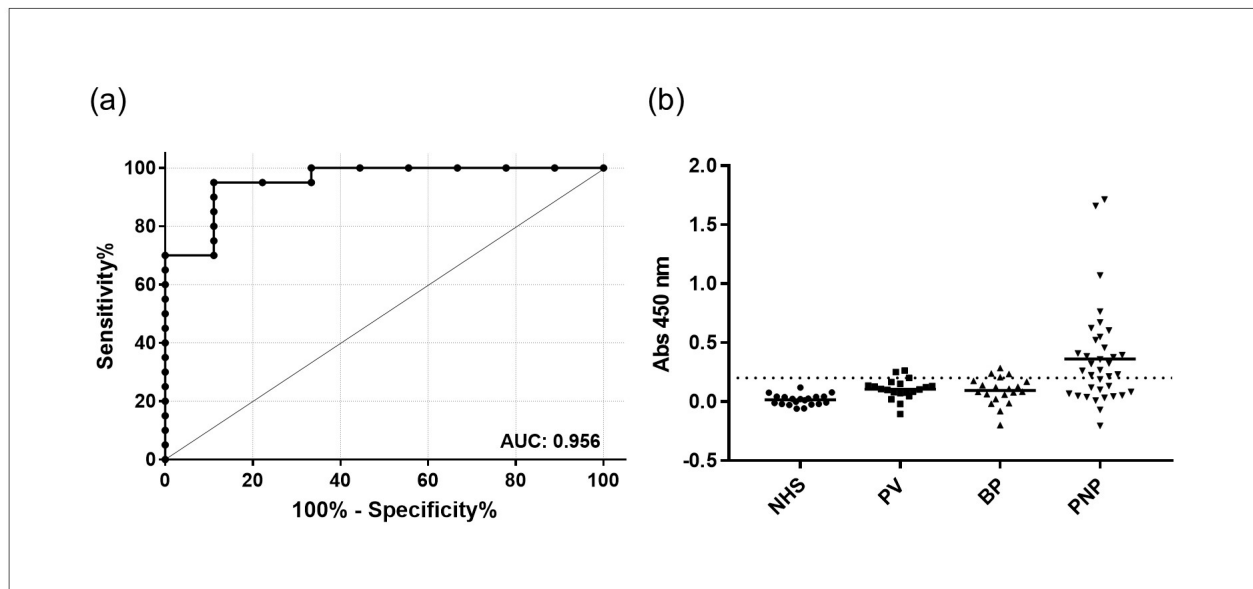


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Supplementary

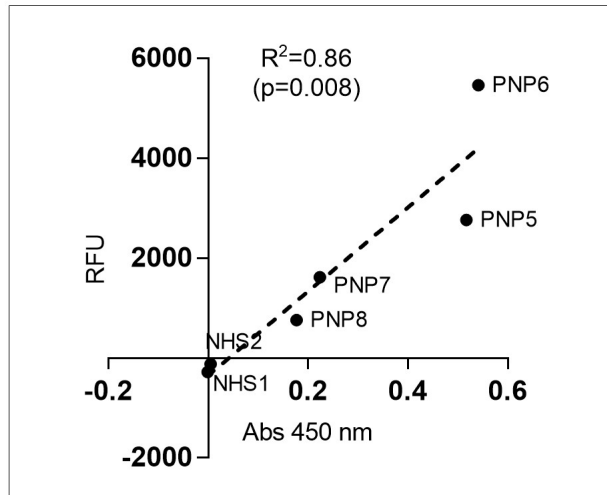


Figure 4. Linear regression model comparing immunoprecipitation (IP) and ELISA based on EGFP-A2ML1. Four PNP sera (PNP5, 6, 7, 8) and two normal sera (NHS1, NHS2) were analyzed by IP and ELISA based on EGFP-A2ML1. Absorbance values at 450 nm are plotted on the x-axis while relative fluorescence units (RFU) are plotted on the y-axis. The coefficient of determination (R^2) and the p-value are indicated.

Supplementary

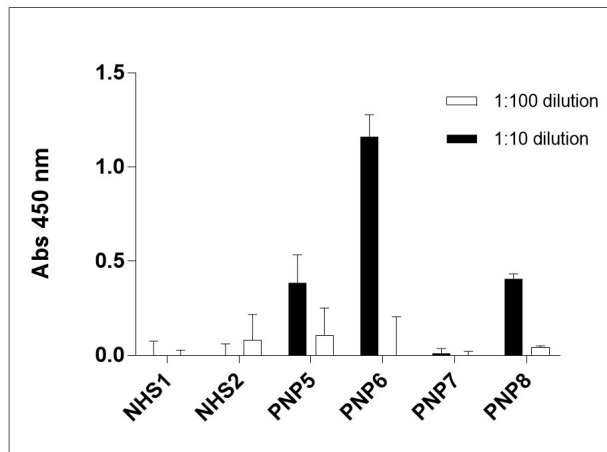


Figure 5. EGFP-A2ML1 enzyme-linked immunosorbent assay with two different sera dilutions.

Four PNP sera (PNP5, 6, 7, 8) and two normal sera (NHS1, NHS2) were analyzed by the ELISA based on EGFP-A2ML1. Two sera dilutions were tested (1:10 and 1:100) and absorbance was measured at 450 nm. The sEGFP background was subtracted from the EGFP-A2ML1 values.

Data are representative of one independent experiment performed in triplicate.